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Enalapril reduces collagen type IV synthesis and expansion of the interstitium in the obstructed rat kidney

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Enalapril reduces collagen type IV synthesis and expansion of the interstitium in the obstructed rat kidney. Chronic unilateral ureteral obstruction (UUO) results in interstitial fibrosis of the affected kidney. In this study we determined that enalapril ameliorates the increased production of extracellular matrix (ECM) protein in the tubulointerstitium during UUO. The relative volume (Vv) of the tubulointerstitium measured by a point-counting method increased significantly at three or five days of UUO as compared to the contralateral kidney. Enalapril significantly blunted this increase at either three or five days. Immunofluorescence studies revealed that collagen type IV increased remarkably in both the tubular basement membrane (TBM) and the interstitial space at three or five days of UUO. Glomeruli did not show any change. Collagen types I and III were faintly stained in the control kidneys while they were obviously increased in the interstitial space of the obstructed kidney. We examined the expression of collagen type IV (COL IV) because this basement membrane matrix protein appeared to be a major ECM protein deposited in the tubulointerstitium of the obstructed kidney. Semiquantitative analysis of COL IV by immunofluorescence microscopy revealed that enalapril reduced slightly (21%) but significantly ($P < 0.01$) the deposition of COL IV in the obstructed kidney. Measurement of cyanogen bromide peptides from the obstructed kidney by Western blotting showed an increase of COL IV. This increase was reduced slightly (20%) by enalapril. The level of COL IV mRNA measured by reverse transcription-PCR was very low or undetectable in the control and contralateral kidneys, while it was significantly increased in the obstructed kidney at three or five days of UUO. COL IV mRNA was abundant in glomeruli while it was almost undetectable in renal tubules in the control and contralateral kidneys. However, COL IV mRNA was increased in renal tubules but not in the glomeruli of the obstructed kidney. Enalapril treatment resulted in a 42% decrease ($P < 0.01$) in COL IV mRNA in the cortex and a remarkable decrease in the renal tubules of the obstructed kidney at five days. Enalapril treatment resulted in an 89% decrease in the number of infiltrating ED-1 positive monocytes/macrophages. These results indicate that enalapril treatment ameliorates the tubulointerstitial fibrosis of the affected kidney in UUO. This effect of enalapril on fibrosis may be due to the severe reduction in monocytes/macrophages capable of secreting the profibrotic factor TGF-β1.

Chronic ureteral obstruction causes tubulointerstitial fibrosis of the kidney. Nagel and Bulger [1] showed that in the rabbit with unilateral ureteral obstruction (UUO) collagen fibers and fibroblasts were increased in the interstitium of the affected kidney at seven days post-obstruction. Sharma et al [2] reported increased kidney synthesis of various ECM components (collagen types I, III and IV, fibronectin and heparan sulfate proteoglycan) in the interstitium of rabbits with ureteral obstruction of three and seven days duration. These data suggest that renal interstitial fibrosis may begin promptly after the onset of obstruction.

TGF-β is a major cytokine in the regulation of production and deposition of ECM proteins [3, 4]. TGF-β regulates gene transcription of collagen types I, III, and IV, fibronectin and laminin [5–7]. We found increased mRNA expression of TGF-β1 in the cortex of the obstructed kidney when compared to the contralateral kidney of rats as early as three days after UUO was initiated [8]. The administration of enalapril, an angiotensin I converting enzyme inhibitor (ACEI), significantly blunted but did not completely abrogate the increased expression of TGF-β1 mRNA present in the renal cortex at five days of UUO [8]. Enalapril reduces glomerular injury in rats with a remnant kidney [9–11]. Enalapril also ameliorates interstitial fibrosis of rats with cyclosporine nephrosis [12], aminonucleoside nephrosis [13] or subtotal nephrectomy [14]. These data suggest that enalapril may have a role in suppressing the increased synthesis of ECM proteins during renal injury although its mechanisms have not been elucidated.

In the present study we focused on the expression of mRNA and protein synthesis of COL IV. This basement membrane matrix protein appeared to be a major component of tubulointerstitial fibrosis during UUO. The previously noted reduction in TGF-β1 mRNA expression due to enalapril treatment [8] was explored by determining the effect of ACEI on monocyte/macrophage infiltration of the UUO kidney. Thus, the aims of this study were to assess mRNA expression of COL IV and its deposition in the tubulointerstitium and to determine whether enalapril ameliorates the increased synthesis of COL IV in the tubulointerstitium of the affected kidney in UUO.

Methods

Experimental protocol

Female Sprague-Dawley rats, weighing 200 to 240 g, were used. Under fluothane anesthesia the left ureter was ligated with 4-0 silk at two points and cut between the ligatures in order

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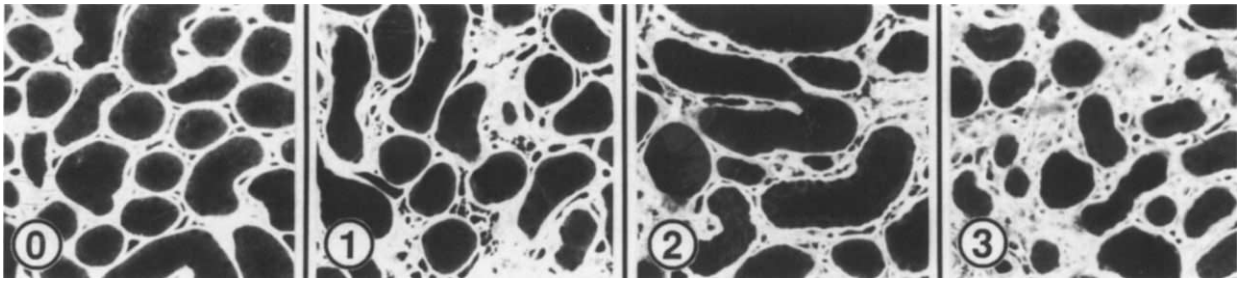

Fig. 1. *Criteria for matrix deposition score for COL IV by immunofluorescence.* Matrix deposition score is determined to be: 0, no changes (control or contralateral kidney); 1, mild change; 2, moderate change; and 3: severe change. Magnification: ×400.

to prevent retrograde urinary tract infection. Sham-operated rats had their ureters manipulated but not ligated. Rats were sacrificed at three or five days of UUO under pentobarbital anesthesia (50 mg/kg body weight, i.p.). Sham-operated rats and unoperated rats were used to obtain "control" kidneys.

Enalapril (5 mg/kg body weight) (Sigma, St. Louis, Missouri, USA), an ACEI, was administered intraperitoneally at 24 hours, 12 hours and one hour before surgery and every 12 hours after surgery for three or five days, at which point the animals were sacrificed. Sham-operated rats were similarly treated and sacrificed at three or five days after surgery.

Tissue preparation

Rats were anesthetized with pentobarbital. For histological studies kidneys were perfused *in vivo* with phosphate buffered saline (PBS), pH 7.2, to clear blood-borne cells and then with 4% paraformaldehyde in PBS, pH 7.2, for 10 minutes. In some rats Histochoice (Amresco, Solon, Ohio, USA) was substituted for paraformaldehyde. The kidneys were removed quickly and sliced longitudinally in 2 mm thick sections, further immersed in the same fixative for four hours at 4°C, washed with PBS and embedded in paraffin. Paraffin sections, 4 μm in thickness, were subjected to Azan-Mallory stain or indirect immunofluorescence stain.

For RNA extraction rat kidneys were perfused *in situ* with cold Hanks' balanced salt solution (HBSS), removed quickly and placed in cold HBSS. The cortex was immediately dissected from the kidney and stored at −70°C. Glomeruli and tubules were isolated by a sieve technique (mesh size: 250, 150 and 75 μm) and stored at −70°C.

Immunofluorescence studies

The deparaffinized sections were washed in 0.05 M Tris-HCl buffer, pH 7.6, and treated with 0.1% trypsin type I (Sigma) in 0.1% $CaCl_2$ and 0.05 M Tris-HCl, pH 7.6 for 30 minutes at room temperature. Sections were washed in PBS for 10 minutes three times, preincubated in blocking solution (10% goat or rabbit serum in PBS) for 30 minutes, washed in PBS three times and then incubated with primary antibody in PBS for two hours at room temperature. Goat polyclonal antibody against type IV collagen (1:40 dilution) (Fisher, Pittsburgh, Pennsylvania, USA), or rabbit polyclonal antibodies against type III (1:40) and type I (1:20) collagen (Chemicon, Temecula, California, USA) were used as primary antibodies. The serum of animals in which the primary antibody was developed was used as a negative control. Each section was washed three times in PBS and incubated with a second antibody (1:80 dilution) in PBS for 30 minutes. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG or goat anti-rabbit IgG (Sigma) was used as a second antibody. After washing with PBS three times, the section was mounted with Fluoromount-G (Fisher, Pittsburgh, Pennsylvania, USA).

Semiquantitative analysis of COL IV by immunofluorescence microscopy

The deposition of COL IV in the tubulointerstitium of renal cortex was assessed semiquantitatively by fluorescence microscopy. The matrix deposition score from 0 to 3 was based on the intensity and distribution of COL IV in the tubulointerstitium: 0, no changes; 1, mild change; 2, moderate change; and 3, severe change (Fig. 1). In each section the matrix score was counted randomly in over 100 fields under high power (×400). Observers did not know whether histologic sections being examined were from control rats or rats with UUO.

Morphometric analysis of the interstitial volume

A standard point counting method was used to quantitate the volume of the renal interstitium [15]. The relative volume (Vv) of the interstitium of the cortex was determined on the sections stained using the Azan-Mallory method to stain collagen fibers in tubular basement membrane (TBM), glomeruli and interstitial space. Under high magnification (×400) consecutive non-overlapping fields were photographed from each section of renal cortex. A grid containing 117 (13 × 9) sampling points was superimposed on each photograph and a total of 1,404 to 2,691 points were evaluated in each kidney. The number of points overlying TBMs and interstitial space were counted while points falling on Bowman's capsule or peritubular capillaries were not. Points falling on glomerular structures or on larger vessels were excluded from the total count.

Immunoblotting for COL IV

SDS-PAGE was performed to quantitate the production of collagen proteins. Collagen fibers in the cortex were cleaved with cyanogen bromide (CNBr) [16, 17]. Renal cortical tissue which had been perfused with HBSS and stored at −70°C was incubated in 70% formic acid at 37°C overnight in screw-cap tubes. CNBr (Sigma) dissolved in 70% formic acid was added to each sample. The sample was flushed with nitrogen to remove oxygen and incubated for four hours at 37°C. The specimen was centrifuged at 35,000 × g for 30 minutes and the supernatant

passed over a column of P6 gel (50 to 100 mesh, Bio-Rad, Richmond, California, USA) (column buffer: 1 M acetic acid) to separate peptides from the formic acid and CNBr. CNBr peptides were lyophilized. The lyophilized protein was dissolved in distilled water, centrifuged briefly and the supernatant processed for immunoblotting. CNBr peptides were electrophoresed through a 13.5% polyacrylamide gel (30% acrylamide/0.4% Bis-acrylamide) containing SDS and transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad) at 100 volts for 60 minutes. The membranes were blocked and exposed to goat polyclonal antibody against COL IV (Fisher) at 1:200 dilution for one hour. Rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma) was used as a second antibody at a 1:10,000 dilution.

RNA extraction

Total RNA was extracted from the renal cortex, glomeruli and tubules using the guanidinium isothiocyanate (RNAzol) method (Cinna/Bioteck, Houston, Texas, USA) [18]. The total RNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA) and quantitated by UV spectrophotometry at 260 nm and 280 nm. RNA with an OD_{260}/OD_{280} ratio over 1.9 was used for cDNA synthesis.

Characterization and quantification of collagen α(IV) mRNA by RT-PCR

First-strand cDNA was synthesized with a cDNA cycle kit from Invitrogen (San Diego, California, USA), using 4 μg of total RNA and priming with oligo dT. The mixture of total RNA and oligo dT primer was incubated at 65°C for two minutes to denature the secondary strand and chilled on ice. The resulting mixture was incubated at 42°C for one hour, heated to 94°C for three minutes and chilled on ice. A second round of reverse transcription was performed after adding 5 units of avian myeloblastosis virus (AMV) enzyme.

Polymerase chain reaction (PCR) coupled to reverse transcription of RNA (RT-PCR) was carried out. Three microliters of each cDNA were amplified to a total volume of 50 μl containing PCR buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 100 pmol of oligonucleotide primers for COL IV or 12.5 pmol of GAPDH primers and 1.25 units of Taq DNA polymerase (Promega, Madison, Wisconsin, USA). In order to quantitate PCR products comparatively and confirm the integrity of the RNAs we coamplified a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a companion tube. We used a small amount (0.5 μl) of cDNA from the cortex to amplify the GAPDH message to prevent the PCR product of GAPDH from reaching a plateau at higher cycles of amplification. The sequences of primers for collagen α1(IV) mRNA were designed from the sequence of the mouse gene: (sense) 5′-GTGCGGTTTGTGAAGCACCG, (anti-sense) 3′-GTTCTTCTCATGCACACTT (363 bp) [19]. The GAPDH primers were designed from the rat gene: (sense) 5-AATGCATCCTGCACCACCAA, (anti-sense) 3′-GTAGCCATATTCATTGTCATA (515 bp) [20]. PCR was carried out with a Perkin Elmer Cetus DNA Thermal Cycler using the following program: 94°C for one minute (denaturing), 54°C for one minute (annealing) and 72°C for two minutes (extension). Amplification was carried out at 45 cycles for the cDNA of cortex and at 40 cycles for the cDNAs of glomeruli and tubules. PCR products were sequenced using the TA Cloning System (Invitrogen) and the AmpliTaq Sequence kit (Perkin Elmer, Norwalk, Connecticut, USA). The resultant sequences (not shown) confirm the identity of the PCR products as collagen α1(IV) and GAPDH.

PCR products were quantitatively analyzed as described previously [8]. After amplification, 15 μl of each PCR reaction mix was electrophoresed through a 1.2% agarose gel with ethidium bromide (0.5 μg/ml). The gel was photographed with Polaroid Type 665 positive/negative film (Polariod Corporation, Cambridge, Massachusetts) over UV light at the same exposure and developing time. The bands on the negative film were scanned by densitometry (Sepra Scan 2001 software, Integrated Separations System, Natwick, Massachusetts, USA) for quantification. The PCR products for collagen α1(IV) or GAPDH amplified from the same cDNA were electrophoresed in the same gel and the ratio of collagen α1(IV)/GAPDH was determined to eliminate gel-to-gel or film-to-film variance.

Identification and quantitation of infiltrating monocytes/macrophages

Histochoice-fixed paraffin embedded sections were used. Monocytes/macrophages were identified using antibody ED-1 (Harlan Bioproducts for Science, Inc., Indianapolis, Indiana, USA) using techniques previously described [21]. The primary antibody was located with an alkaline phosphatase-linked goat antimouse IgG (Sigma) and Fast Red color development. Hematoxylin was used as the counterstain. The number of ED-1 positive cells was determined in three randomly chosen 400× fields within the same section of renal cortex from an individual animal and averaged. The average number of ED-1 positive cells from three separate animals was calculated.

Blood pressure measurement

Measurements were obtained as previously described [22]. Briefly, rats were lightly anesthetized with halothane. An indwelling PE-50 catheter was placed into the left femoral artery. After 30 minutes for recovery from anesthesia and surgery, three successive readings were obtained. Mean arterial pressure (MAP) was determined continuously through the left femoral artery catheter connected to an electronic transducer (Weco VT-1, Winston Electronics, Millbrae, California, USA).

Statistical analysis

Data, shown as mean ± SD, were analyzed by the unpaired *t*-test. An unpaired *t*-test with Welch's correction was used to analyze the data on the relative volume of the renal interstitium and blood pressure. ANOVA was employed to determine the significance of the ED-1 cell infiltrate into the renal cortex.

Results

Morphology of the renal cortex during UUO

Figure 2 demonstrates sections of renal cortex stained with Azan-Mallory stain. In the control kidney collagen fibers were observed in the TBM, Bowman's capsule, and mesangium and around the intertubular capillaries. The peritubular interstitial space was usually very narrow. Slightly wider spaces were also seen, characterized by interstitial cells and small amounts of

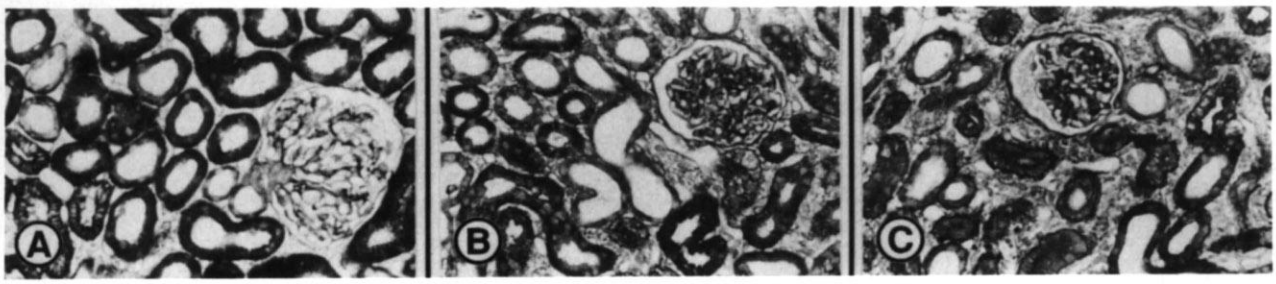

Fig. 2. *Photographs of renal cortex stained with Azan-Mallory.* Control kidney (A) and obstructed kidneys at 3 days (B) and 5 days (C) after UUO are shown. The contralateral kidney showed no difference from the control kidney on both days. Magnification: ×400.

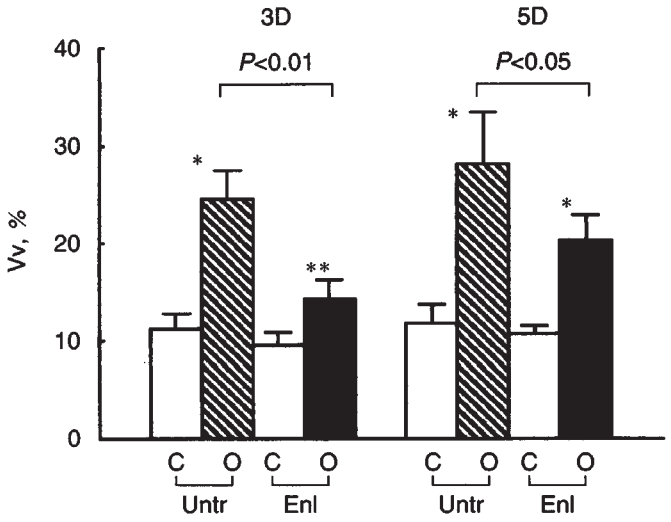

Fig. 3. *Volume fraction (Vv) of the tubulointerstitium with or without enalapril treatment during UUO.* Abbreviations are: C, contralateral kidney; O, obstructed kidney; UNTR, untreated; ENL, enalapril. At 3 days $N = 4$ for untreated animals and $N = 3$ for enalapril-treated animals. At 5 days $N = 5$ for each group. The bar represents mean ± SD. * $P < 0.01$; ** $P < 0.05$ versus contralateral kidneys.

collagen fibers (Fig. 2A). On the third day of UUO the affected kidney showed an obviously widened interstitial space with a greater number of interstitial cells and infiltrating leukocytes (Fig. 2B). Collagen fibers were increased in the interstitial space, forming a fine network. Thickening of the TBM was beginning in some renal tubules but this was a minor change at three days. At five days collagen fibers were further increased in the interstitial space. The TBM was thickened in the area where interstitial inflammation was more prominent (Fig. 2C). These changes were observed in the whole cortex at five days although the degree of severity was not homogeneously distributed. In contrast, there were no changes in collagen deposition in the glomeruli of either the contralateral or obstructed kidney at three or five days.

Morphometric analysis of interstitial volume

The relative volume of the interstitium was expressed by the volume fraction (Vv) (Fig. 3). Vv of the contralateral kidney was approximately 10% (same as that of the control kidney) and did not change with enalapril administration. The interstitial volume of the obstructed kidney was significantly ($P < 0.01$) increased at three days or five days as compared to that of the contralateral kidneys. Enalapril administration blunted the increase in the interstitial volume significantly ($P < 0.01$), from 25% to 14% at three days and from 28% to 20% at five days, although the interstitial volume of the enalapril-treated rats was still significantly greater than that of the contralateral kidney, which averaged 10 to 12%.

Immunofluorescence studies on COL I, COL III and COL IV proteins

In the control and contralateral kidneys of rats with UUO, COL IV was observed very clearly in the TBM, Bowman's capsules, peritubular capillaries and in the mesangium. There was little COL IV in the interstitial space (Fig. 4A). The amount of COL IV in the contralateral kidney did not change at three or five days of UUO as compared to that of the control kidney. The obstructed kidney showed an increased deposition of COL IV at three days. There was thickening of the TBM and a widened interstitial space of the renal cortex. The increase was more prominent at five days (Fig. 4B). Increased deposition of COL IV was observed mainly in basement membranes, Bowman's capsules and peritubular capillaries. However, it was diffusely increased in the interstitial space where the deposition was prominent. The increase of COL IV was found in the whole area of the renal cortex of the obstructed kidney at day 5, although the increase was not homogeneously observed. Glomeruli did not show any change in COL IV deposition in either the contralateral or the obstructed kidney.

COL III was faintly stained and was confined to the renal interstitial space and the mesangium. It was not detected in the TBM in the control and contralateral kidneys (Fig. 4C). In the obstructed kidney COL III was obviously increased in the interstitial space surrounding tubules and capillaries and formed a fine network in the interstitium although the intensity of immunostaining was still low (Fig. 4D).

COL I was well stained when the kidney was fixed with Histochoice but not with 4% paraformaldehyde. COL I, similar to COL III, was restricted to the interstitium but the amount of COL I was less than that of COL III in the control and contralateral kidneys (Fig. 4E). In the obstructed kidney COL I increased remarkably in the interstitial space (Fig. 4F). Fundamentally COL I was not increased in TBM of the obstructed kidney, although the thickened COL I fibers enclosed the renal tubules giving the appearance of a thickened TBM. COL I and COL III were faintly observed in the glomeruli in the control

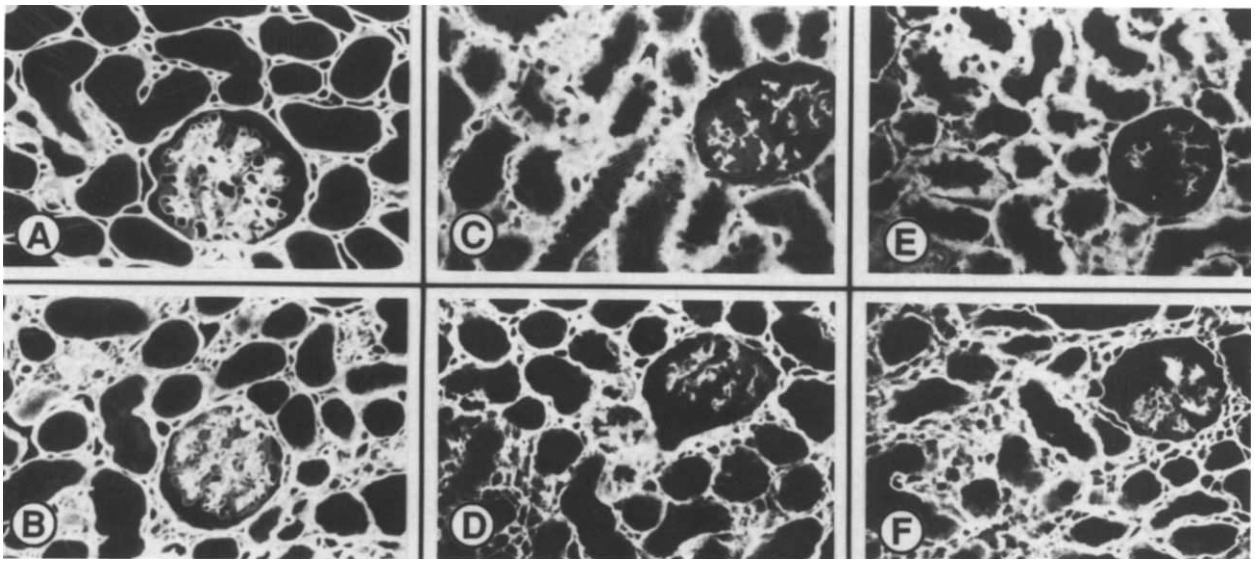

Fig. 4. *Immunofluorescent micrographs of contralateral and obstructed kidneys at 5 days of UUO, stained for COL IV* (**A** *and* **B**, *respectively), COL III* (**C** *and* **D**, *respectively) and COL I* (**E** *and* **F**, *respectively).* Sections fixed with 4% paraformaldehyde were used for COL IV and III and a Histochoice-fixed specimen was used for COL I. Magnification: ×400.

Table 1. Matrix deposition score of COL IV in the obstructed kidney in rats with UUO of 5 days duration

Group	*N*	Matrix score 0	1	2	3
Untreated	3	0	8.9 ± 4.4	40.2 ± 8.5	50.9 ± 10.6
Enalapril	5	2.3 ± 1.9	24.8 ± 7.5[a]	53.5 ± 4.2[a]	22.0 ± 8.1[b]

The relative frequency of matrix score was obtained from over 100 different fields of each separate kidney. Values are means ± SD (%) of relative frequency at each matrix score point. The mean scores of the control and contralateral kidneys were near 0 in every rat.

[a] $P < 0.05$ and [b] $P < 0.01$ when compared to the relative frequency of untreated rats at the same matrix score point

and the contralateral kidneys. However, glomeruli did not show any increase of these proteins during UUO.

Matrix deposition score of COL IV

We determined the matrix deposition score of COL IV from 0 to 3 in order to assess whether enalapril treatment affected the increased deposition of COL IV in the obstructed kidney at five days of UUO. The mean score of the control and contralateral kidneys was near 0 in every rat (data not shown). The relative frequency of each score point was obtained from over 100 different fields of each kidney (Table 1). The untreated rats showed a higher frequency at point 3 and a lower frequency at points 0 and 1. However, in the enalapril treated rats the frequency of points 1 and 2 increased significantly and that of point 3 decreased significantly when compared to the data for the untreated rats. Figure 5 illustrates the mean matrix score of each group. The mean score of the untreated rats (2.4 ± 0.1) was slightly but significantly ($P < 0.01$) decreased, to 1.9 ± 0.2, by enalapril treatment although the score was still high after treatment when compared to the score of control kidneys or contralateral kidney of rats with UUO.

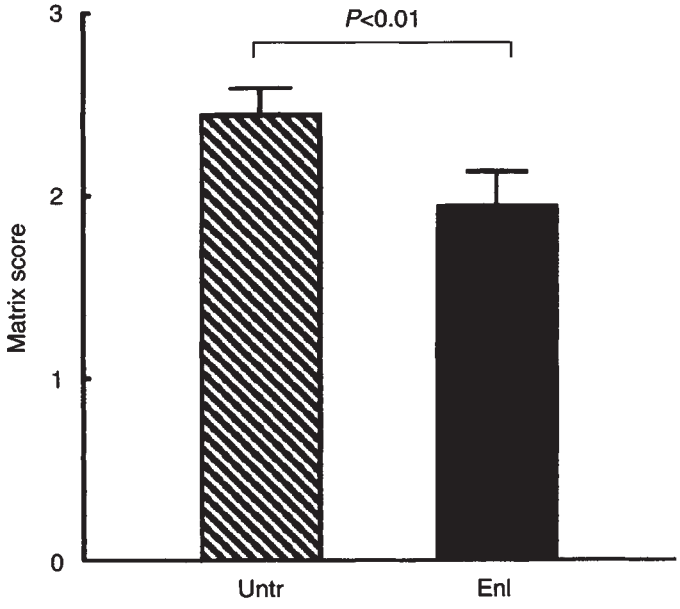

Fig. 5. *Mean matrix deposition score of COL IV from the obstructed kidney of untreated (UNTR,* N = *3) and enalapril (ENL,* N = *5) untreated rats.* The bar represents mean ± SD.

Immunoblotting of CNBr peptides against COL IV

The immunoblot depicted in Figure 6 shows several CNBr peptides from COL IV in the cortex at five days of UUO. Collagen proteins were cleaved into several smaller weight peptides by CNBr. Peptides of 84 kDa and 73 kDa were immunogenic against the COL IV antisera used. These CNBr peptides were obviously increased in the renal cortex of the obstructed kidneys compared to the control and the contralateral kidneys. The amount of COL IV peptides in the cortex of the obstructed kidney was slightly decreased by enalapril.

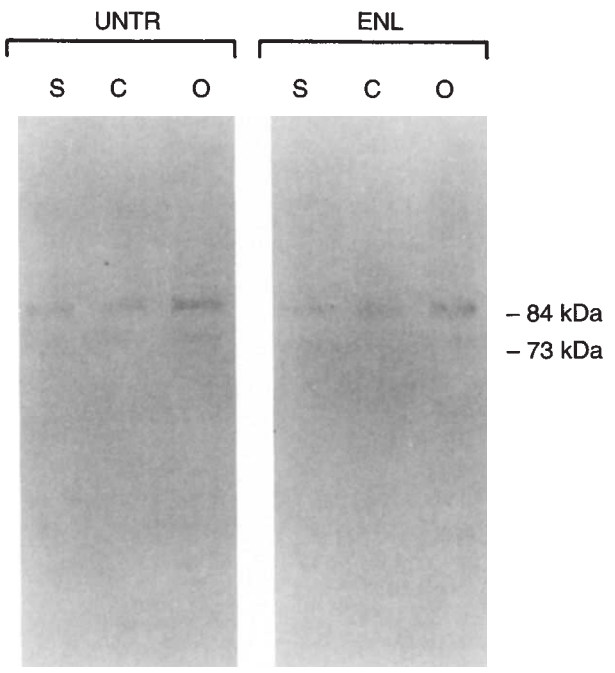

Fig. 6. *Immunoblotting of CNBr peptides against COL IV in the cortex at 5 days of UUO.* Twenty micrograms of CNBr peptides was applied to each lane. Numbers represent molecular weights of pre-stained molecular standards. Abbreviations are: UNTR, untreated; ENL, enalapril; S, sham-operated kidney; C, contralateral kidney; O, obstructed kidney.

Densitometric analysis revealed an approximate 20% decrease in the amount of CNBr peptides in the obstructed kidney of rats treated with enalapril.

mRNA expression of collagen α1(IV) during UUO

Figure 7A demonstrates the PCR products of collagen α(IV) and GAPDH at three or five days of UUO. A PCR product for collagen α1(IV) showed a very faint band or was not detectable in the contralateral kidney at every time point. The obstructed kidney showed a prominent increase of mRNA level at three days and the level was still high at five days of UUO. The GAPDH PCR products were prominent and not significantly different in renal cortex of sham, contralateral or obstructed kidneys. We measured mRNA expression of collagen α1(IV) in isolated glomeruli and tubules from the kidney after five days of UUO to determine the region of the nephron in which the matrix protein mRNA might be increased (Fig. 7B). We found that the mRNA of collagen α1(IV) was abundant in glomeruli of the contralateral kidney and that the mRNA level did not change in the glomeruli of obstructed kidney during UUO. However, the renal tubules showed a prominent increase of mRNA expression in the obstructed kidney while mRNA expression was very low or undetectable in the contralateral kidney. Figure 8 demonstrates the relative level of mRNA expression of collagen α1(IV) in the obstructed kidney at three or five days of UUO. The amount of mRNA of the contralateral kidney was essentially undetectable (data not shown). The obstructed kidney showed a remarkable increase in mRNA expression at either three or five days of UUO. The increased mRNA level of collagen α1(IV) present in the obstructed kidney

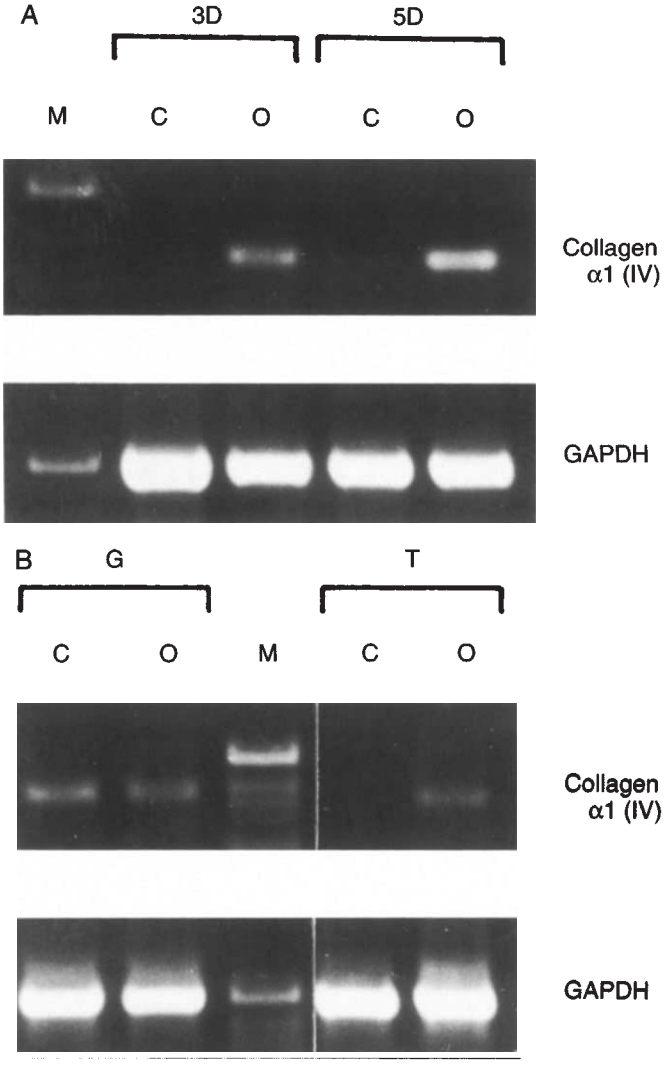

Fig. 7. *mRNA expression of collagen α1(IV) in the cortex* **(A)** *at 3 or 5 days and in the glomeruli and tubules* **(B)** *at 5 days of UUO.* Abbreviations are: C, contralateral kidney; O, obstructed kidney; M, DNA standard marker; G, glomeruli; T, tubules.

was significantly ($P < 0.01$) blunted (42%) but not completely suppressed by enalapril treatment at five days, while it was not affected at three days of UUO.

We previously showed that TGF-β1 mRNA increased in renal cortical tubules rather than in the glomeruli obtained from the obstructed kidney at five days of UUO [8]. In the present study we measured the expression of collagen α(IV) and TGF-β1 in the renal tubules from rats with or without enalapril treatment. This was to determine whether the increase in mRNAs present in the tubules of the obstructed kidney was suppressed by enalapril. PCR amplification was performed using 2 μg of RNA and 30 cycles for TGF-β1 as described previously [8]. Figure 9 demonstrates PCR products from two different experimental animals following five days of UUO. In the untreated rats both collagen α1(IV) and TGF-β1 mRNAs were increased in the obstructed kidneys when compared to the contralateral kidneys. In contrast, in the enalapril-treated rats collagen α(IV) mRNA was undetectable in the obstructed

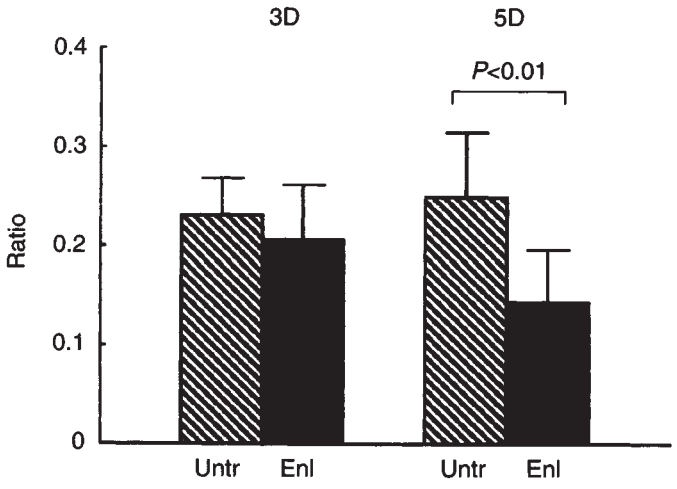

Fig. 8. *Relative level of mRNA of collagen α1(IV) in the obstructed kidney with (ENL) or without (UNTR) enalapril treatment at 3 or 5 days of UUO.* The ratio on the vertical axis refers to COL IV/GAPDH. The bar represents mean ± SD. UNTR: $N = 4$ and 6 at 3 and 5 days of UUO, respectively. ENL: $N = 7$ at 3 or 5 days.

kidneys and TGF-β1 mRNA was suppressed both in the contralateral and obstructed kidneys.

Blood pressure

Enalapril-treated rats showed significantly ($P < 0.01$, unpaired t-test with Welch's correction, $N = 3$) lower MAP than untreated rats at three days and five days of UUO (110 ± 1 vs. 121 ± 2, 100 ± 1 vs. 124 ± 2, respectively). Mean arterial pressures of untreated rats at three days and five days of UUO were not different from those of control rats (121 ± 1).

Infiltration of the kidney by monocytes/macrophages during UUO

As has been reported previously [21], there is an infiltration of the renal cortex by ED-1 positive monocytes/macrophages (Fig. 10A). The infiltrate at day 5 of UUO is larger than that previously reported at day 2 of UUO [21]. In contrast, enalapril pretreatment and cotreatment of the animals resulted in a significant reduction in the number of infiltrating ED-1 positive cells (Fig. 10B and Table 2). There was no significant infiltrate of ED-1 positive cells in the contralateral kidney of rats with UUO compared to the kidney of normal rats (not shown). Enalapril treatment had no discernible effect on the monocyte/macrophage content of the contralateral kidney.

Discussion

Nagel and Bulger [1] reported that in the obstructed kidney of rabbits the interstitial space was widened and interstitial fibrosis was present at seven days after UUO. We found that after three days of UUO the relative volume of the cortical interstitium is increased significantly in the obstructed kidney of the rat. The deposition of COL I, COL III and COL IV also increased in the tubulointerstitium by the third day of UUO. Furthermore, the mRNA level of collagen α1(IV) was significantly elevated in the obstructed kidney at this time point. Thus, interstitial fibrosis is initiated promptly after the onset of obstruction. We demonstrated by immunofluorescence studies that the amount of COL I, COL III and COL IV in the glomeruli of the obstructed kidney was not changed at 5 days of UUO. Our previous study showed that TGF-β1 mRNA levels did not change in the glomeruli of the obstructed kidney during UUO [8]. These data are consistent with the fact that glomeruli appeared normal by light microscopy through 7 days of obstructive nephropathy [22, 23]. The localization of interstitial COL I and COL III in the glomeruli of the normal kidney is controversial [24–27]. However, we found COL I and COL III in the glomeruli of our specimens of rat kidneys when fixed with Histochoice and when the sections were treated with trypsin to uncover these epitopes.

We also found that both the interstitial collagen (types I and III) and the basement membrane collagen (type IV) were deposited in the interstitial space of the obstructed kidney. In the obstructed kidney both COL I and COL III were increased in the interstitial space only, while COL IV was deposited both in TBM and the interstitium of the obstructed kidney. Haralambous-Gasser et al [28] quantitated collagen by CNBr peptide analysis in the whole kidney of newborn rats with incomplete UUO. They showed that over 50% of the collagen in the normal kidney was COL I and COL III and these appeared to be major collagens that were increased in the obstructed kidney of newborn rats. Our immunofluorescence studies demonstrated that COL IV is a major collagen that was increased in the renal cortex of adult rats during UUO. The increase in COL IV in both TBM and interstitial space may contribute to the dysfunction of renal tubules of the obstructed kidney. Thus, we focused mainly on collagen IV as a global indicator of fibrosis within the kidney even though the effects of other TBM or ECM proteins in the setting of obstructive nephropathy may be considerable. Jones et al [24] showed that during purine aminonucleoside nephrosis COL IV increased in both TBM and the interstitium; COL I and COL III levels increased but were confined to the interstitium. Downer, Phan and Wiggins [25] reported that in a model of crescentic nephritis in the rabbit COL IV was localized to glomeruli and the periglomerular area while COL I and COL III were present in both the glomeruli and the interstitium. The meaning of the difference of deposition patterns between COL I/III and COL IV is not clear. Intrinsic differences related to the renal injuries may be partially explanatory. It should be noted that there is a monocyte/macrophage influx into the glomeruli of aminonucleoside nephrosis [24, 29] that is absent from glomeruli of obstructed kidneys [21].

Cultured renal tubular cells produce collagen types I, III and IV [30, 31]. We found that the expression of collagen α1(IV) mRNA was remarkably increased in the tubules of the obstructed kidney. Thus, it is conceivable that renal tubules contribute to the increased production of COL IV and the increased deposition of COL IV in both TBM and interstitium. Nagel and Bulger have reported that fibroblasts migrated and proliferated in the interstitium of the obstructed kidney during UUO [1] and that interstitial fibroblasts produce COL I, III and IV [3]. Kuncio, Neilson and Haverty have observed that several cytokines secreted by infiltrating macrophages and T-lymphocytes act as chemoattractants and stimulate fibroblast proliferation [3]. The remarkable increase in COL I and COL III that we found in the interstitium of the obstructed kidney at three or five days of UUO is consistent with the increased

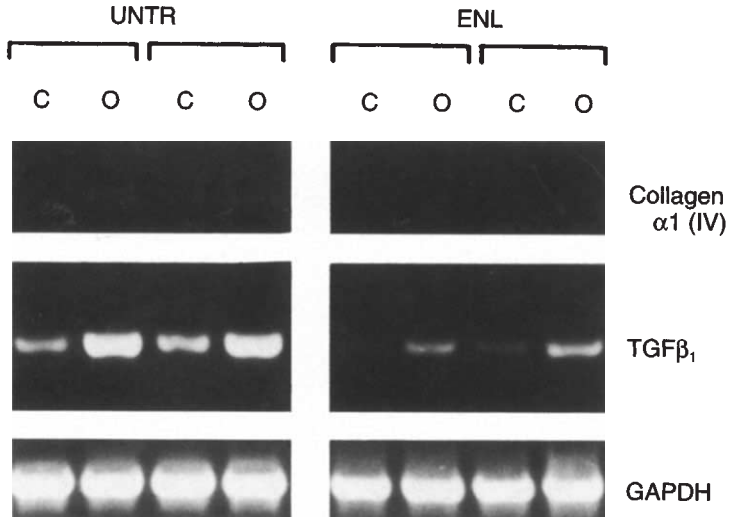

Fig. 9. *Effects of enalapril on mRNA expression of collagen α1(IV) and TGF-β1 in the renal cortical tubules after 5 days of UUO.* Results are from two different experimental animals of untreated (UNTR) or enalapril-treated (ENL) group. Abbreviations are: C, contralateral kidney; O, obstructed kidney.

cellularity due to fibroblast proliferation and infiltrating mononuclear cells. Thus, the interstitial fibroblasts may also contribute to an increase in production of collagens in the obstructed kidney in UUO.

We reported previously that TGF-β1 mRNA expression increased in the obstructed kidney and that this increase was found in renal tubular cells rather than glomeruli [8]. TGF-β1 has pleiotropic effects on matrix protein production [32]. These include an increase in the mRNA of ECM components, particularly the collagens [3, 32], a decrease in proteases degrading these proteins and an increase in metalloprotease inhibitors. Our studies suggest that the increase in TGF-β1 in the cortical tubules of the obstructed kidney results in an increase of collagen α1(IV) mRNA. The increase in collagen IV protein is undoubtedly due to the increase in the mRNA. Although we did not measure proteases or proteases inhibitors, they may also have a role in the fibrosis of ureteral obstruction.

We found that enalapril treatment significantly blunted the increased volume of the interstitium present in the obstructed kidney at either three or five days of UUO. Enalapril curtailed the increased amount of COL IV in the obstructed kidney as assessed by the matrix deposition score (Table 1) and by immunoblotting of CNBr peptides of the whole renal cortex (Fig. 6). The increase in collagen α1(IV) mRNA level was significantly blunted by enalapril at five days but not at three days of UUO. Thus, the decrease of COL IV deposition might be due to the decrease of mRNA expression in renal cortex although this would not account for the reduction in relative volume of the interstitium at three days. It may be that other types of collagen or other extracellular matrix components are decreased by enalapril. Enalapril might also affect the balance between production and degradation of ECM proteins. Enalapril was found to significantly decrease the number of infiltrating ED-1 positive monocytes/macrophages into the interstitium. This would decrease the cellularity within the interstitium and contribute, in part, to the blunted volume increase of this compartment. Effects of enalapril on fibroblast proliferation remain to be determined.

The dosage of enalapril we injected intraperitonealy is essentially equivalent to 200 mg/liter in drinking water based upon the average water consumption by the rat in a 24-hour period. This is higher than the dose (50 mg/liter drinking water) used by others [33]. Ikoma et al [34] showed a greater effect of low dose (50 mg/liter) than high dose (200 mg/liter) ACEI on structure-preservation in a nephrectomy model. They suggested that the low dose of enalapril blocked endothelial ACE and the high dose of ACEI blocked endogenous ACE in kidney. We previously showed that prior inhibition of ACE by enalapril (5 mg/kg body wt twice a day) ameliorated the decreased renal function present in the obstructed kidney [35] and suppressed endogenous angiotensin production during ureteral obstruction [36]. We showed that the high dose of enalapril used in the present study reduced MAP of UUO rats to a significantly lower level as compared to that of control rats and that enalapril ameliorated interstitial fibrosis present in the obstructed kidney. However, there is some evidence that ACEI has greater protective effects on progressive renal injury than triple therapy (reserpine, hydralazine and hydrochlorothiazide) although both therapies normalized systemic hypertension [33]. Further, Kakinuma et al [37] reported that ACEI, not triple therapy, ameliorated vascular lesions of chronic renal failure although both ACEI and triple therapy suppressed the increased blood pressure of nephrectomized rats. In this latter study [37], twice as much enalapril was used as in the present study.

The renin-angiotensin system is activated after ureteral obstruction [38]. In addition, angiotensin II stimulates transcription and synthesis of COL IV in cultured proximal tubular cells [39] and rats infused with angiotensin II develop interstitial fibrosis with an increased COL IV deposition [40]. Renal proximal tubular cells have an active renin-angiotensin operating system [41] while tubular cells produce and secrete angiotensin II into the intratubular fluid [42]. We demonstrated that the expression of collagen α1(IV) mRNA was increased in the renal tubules and this increase was abrogated by enalapril. The increase in TGF-β1 mRNA level present in the obstructed kidney was also suppressed by enalapril (Fig. 9), as was the monocyte/macrophage infiltration (Fig. 10). Angiotensin II, therefore, may contribute to the increased production of COL IV and to a possible chemoattractant to monocytes/macrophages in the obstructed kidney during UUO. The stimulating

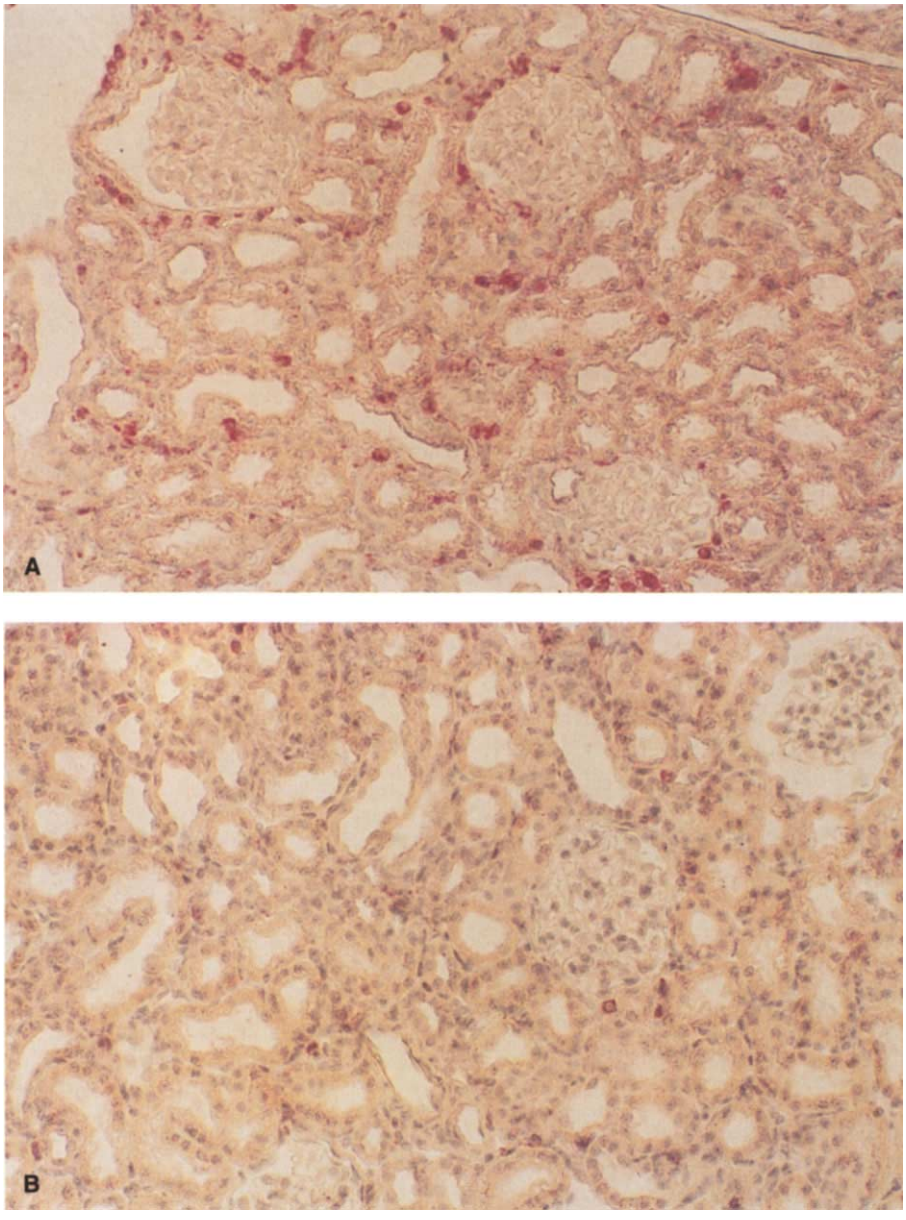

Fig. 10. *Effect of enalapril on the ED-1 positive cell infiltrate in ureteral obstructed rat kidney.* Depicted are representative photomicrographs of cortex of untreated (**A**) and enalapril treated (**B**) animals.

Table 2. Effect of enalapril treatment on the monocyte/macrophage infiltrate of the kidneys of rats with UUO of 5 days duration

Group	Condition	
	Obstructed kidney	Contralateral kidney
Untreated	60.2 ± 5.1[a]	2.9 ± 0.2
Enalapril treated	6.8 ± 1.5	2.5 ± 0.5

Values are means ± SD of the number of ED-1 positive cells per 200× field of kidney cortex of 3 separate animals.

[a] $P < 0.001$ by ANOVA of untreated versus treated obstructed kidney

effects of angiotensin II on COL IV production in the renal tubules may be mediated by an increase in TGF-β1. However, the increased deposition and expression of collagen IV mRNA in the renal cortex were not completely abrogated by enalapril administration. While enalapril effectively reduced the monocyte/macrophage infiltrate within the renal cortex it did not as effectively reduce TGF-β1 mRNA expression [8]. Intrinsic kidney tubular cells probably account, in part, for the increased TGF-β1 mRNA expression due to UUO. The monocyte/macrophage infiltrate may account for the remainder of the TGF-β1 mRNA expression. Thus, other mechanisms probably contribute to the initiation and progression of interstitial fibrosis during obstructive nephropathy.

In summary, we demonstrated that the relative volume of the tubulointerstitium was significantly increased in the renal cortex of the affected kidney at three days or five days of UUO when compared to the control and contralateral kidneys. This increase was due to an increase in the deposition of both interstitial collagens (types I and III) and basement membrane

collagen (type IV). The amount of protein and expression of COL IV mRNA were increased in the obstructed kidney. Enalapril blunted the increase in the interstitial volume, COL IV deposition and COL IV mRNA present in the renal cortex of the obstructed kidney. These results indicate that enalapril substantially ameliorates the tubulointerstitial fibrosis of the affected kidney in UUO. The mechanism by which enalapril retards or inhibits fibrosis may be through a substantial decrease in the number of infiltrating monocytes/macrophages in the UUO kidney. This may contribute, in part, to the decrease in TGF-β1 mRNA expression and eventual profibrotic effect of this factor.

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